abundant re-externalized BSAG particles. PM: plasma membrane. B, RN cells were pulsed with BSAG for 10 min. and chased for 20 min. Ultrathin cryosections were double-immunolabeled with anti-class II antibody and with a monoclonal anti-LAMP1 antibody (31) as indicated. One of two neighboring profiles is shown, exocytotic profile containing BSAG and numerous exosomes labeled for MHC class II and LAMP1.Bars, 0.1 μm.

Figure 2:

Isolation of exosomes from cell culture media. A, RN cells were washed by centrifugation and re-cultured in fresh medium for 2 days. Cell culture media (35 ml) containing 2-5 x10^8 RN cells were centrifuged twice for 10 min. at 300 g (lane 1, first run; lane 2, second run). Lane 1 contains material from 0.6 x 10^6 cells. Membranes in the culture medium from 2-5 x 10^8 cells were pelleted by sequential centrifugation steps: twice at 1200 x g (lane 3 and 4), and once at 10,000 x g (lane 5), 70,000 x g (lane 6) and 100,000 x g (lane 7). The pellets were solubilized at 100°C under reducing conditions and analyzed by Western blotting using [^{125}I]-protein A. Per lane, samples equivalent to 1 x 10^6 cells were loaded. MHC class II α and β chains were recovered mainly from the cells (lane 1) and from the 70,000 x g pellet (lane 6). B, whole mount electron microscopy of the 70,000 x g pellet immunogold labeled for MHC class II. The 70,000 x g pellet was resuspended in RPMI medium, adsorbed to Formvar-carbon coated EM grids, fixed with 0.5% glutaraldehyde in 0.1 M phosphate buffer, immunolabeled with rabbit polyclonal anti-class II antibody and 10 nm PAG and stained using the method described for ultra-thin cryosections (30). The pellet is composed of 60-80 nm vesicles showing abundant MHC class II labeling. Bar, 0.2 μm

Figure 3:

Figure 3A, MHC class II present in the media are membrane bound. Membranes pelleted from culture media at 70,000 x g after differential ultracentrifugation were fractionated by floatation on sucrose gradients, and the non-boiled and non-reduced fractions analyzed by SDS-PAGE and Western blotting with the rabbit polyclonal anti-class II antibody (17). MHC

class II molecules were recovered in fractions 5 to 12 corresponding to densities of 1.22-1.10 g/ml. The majority of MHC class II was in the SDS-stable compact form with a MW of 56-60 kD (Coc/β). Figure 3B, Release of newly synthesized MHC class II molecules. RN cells were pulse-labeled with [^{35}S] methionine for 45 min. (lane 0) followed by chases in the absence of label for 6, 12 and 24 hours. MHC class II molecules were immunoprecipitated from lysates of the cells and pelleted exosomes with the monoclonal DA6.231 anti-class II antibody. RN cells (2 x 10^8) were washed 3 times with ice cold PBS and incubated for 30 min. at 0°C with 1mg/ml Sulfo-NHS-biotin (Pierce). Biotin was quenched for 30 min. with 50 mM NH_4Cl. After washing with ice cold PBS, half of the cells were solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. The remaining biotinylated cells were homogenized. The homogenates were centrifuged and ultracentrifuged identically to the cell culture supernatants and the 70,000 x g pellets solubilized in SDS-sample buffer supplemented with β-mercaptoethanol (control for plasma membrane remnants). Exosome preparations (70,000 x g pellets of cell culture media from 2 x 10^8 cells) were biotinylated as described above and solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. MHC class II was immunoprecipitated from a sample of biotinylated exosomes with the monoclonal anti-class II antibody DA6.231 (19). The biotinylated cell membranes, biotinylated exosomes and immunoprecipitated MHC class II were analyzed by SDS-PAGE and Western blotting with ^{125}I-Streptavidin. Immunoprecipitated MHC class II molecules were dissociated from the sepharose beads at non-reducing conditions at room temperature and analyzed by SDS-PAGE and fluorography. After pulse-labeling (0), MHC class II immunoprecipitated from the cells as SDS-unstable complex of α-β-invariant chain. SDS-stable α-β dimers were recovered from the cells after 6 hours of chase and the signal increased thereafter. In the exosomes pellets SDS-stable αβ dimers started to appear at 12 hours. Figure 3C, Exosomes and plasma membrane display different patterns of biotinylated proteins. In plasma membranes (lane 2) and experimentally produced remnants of plasma membranes many biotinylated proteins are detected with ^{125}I-Streptavidin (lane 5). In exosomes (lanes 3 and 4, show increasing concentrations of exosomes, respectively) two major

proteins with a MW of 60-70 kD are detected. Lane 1 shows the immunoprecipitation of biotinylated class II α and β chains from exosome lysates. In these assay the higher electrophoretic mobility of α and β chains is due to their efficient binding to biotin. Two minor bands at a MW of 200-300 kD are detected in exosomes (lanes 1, 3 and 4, arrows) and are absent from the plasma membrane.

Figure 4:

Presentation of HSP 65 antigen by HLA-DR15 positive RN B cells and exosomes to the $CD4^+$ T cell clone 2F10. Proliferative responses to naive cells Figure 4(A), to cells pre-incubated with antigen Figure 4(B), to exosomes derived from naive cells Figure 4(C) and to exosomes derived from cells pre-incubated with antigen Figure 4(D). The closed symbols show proliferation measurements after addition of HSP 65 derived peptide (418-427), the open symbols where peptide was not added. HLA-class II restriction was determined by adding 10 μg/ml anti-DR antibody (triangles), anti-DP (circles), or no antibody (squares). The exosomes at the highest concentration were derived from media of 1.6×10^6 cells. All assays were performed in triplicate and results are expressed in cpm [^{3}H]-thymidine incorporated into T cells. The SEM for triplicate cpm measurements was less then 10%. Results shown form a representative example of experiments performed in duplicate.--.

On page 2, lines 30 (both occurences) and 37, change "70.000 g" to --70,000 x g--.

On page 3, line 1, delete "(14)" and before "Western" insert the following text:

--The 70,000 x g pellet obtained after differential centrifugation of the cell culture supernatants of RN B lymphoblastoid cells was resuspended in 5 ml of 2.5 M sucrose, 20 mM HEPES/NaOH pH 7.2. A linear sucrose gradient (2 M-0.25 M sucrose, 20 mM HEPES/NaOH, pH 7.2) was layered over the exosome suspension in a SW27 tube and was centrifuged at 100,000 x g for 15 hrs. Gradient fractions (18 x 2 ml) were collected from the bottom of the tube, diluted with 3 ml PBS and ultracentrifuged for 1 hr at 200,000 x g using a SW50 rotor (Beckman). The pellets were solubilized at room temperature in SDS

buffer lacking mercaptoethanol and analyzed by SDS-PAGE and Western blotting using ^{125}I-Protein A.--.

On page 3, line 1, start a new paragraph with the word "Western".

On page 3, line 9, start a new paragraph with the word "To".

On page 3, line 14, delete "(16)" and before "After" insert the following text:

--RN cell were pulsed for 45 min. with 50 Mbq/ml [^{35}S]-methionine (Tran-Slabel, ICN, CA) and chased for different periods of time (5 x 10^7 cells per time point). After pulse-chase labeling, the cells were pelleted by centrifugation for 10 min. at 300 x g. The supernatants were collected and centrifuged for 5 min. at 10,000 x g and then for 30 min. at 200,000 x g pellets were lysed and MHC class II and TfR were immunoprecipitated from equal samples of the lysates. TfR was immunoprecipitated from equal samples of the lysates. TfR was immunoprecipitated as described previously (16). MHC class II was quantitated using a Phosphoimager.--.

On page 3, line 15, change "α-β-l-chains" to --α-β-I-chain--.

On page 3, line 27, start a new paragraph with the word "To".

On page 3, lines 30, 31, 33, 35 and 37, change "biotinilated" to --biotinylated--.

On page 3, line 32, change "^{125}I-Streptavidin" to --^{125}I-Streptavidin--.

On page 3, line 32, delete "(18)", and before "Figure" insert the following text:

--RN cells (2 x 10^8) were washed 3 times with ice cold PBS and incubated for 30 min. at 0°C with 1 mg/ml Sulfo-NHS-biotin (Pierce). Biotin was quenched for 30 min. with 50 mM NH_4Cl. After washing with ice cold PBS, half of the cells were solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. The remaining biotinylated cells were homogenized. The homogenates were centrifuged and ultracentrifuged identically to the cell culture supernatants and the 70,000 x g pellets solubilized in SDS-sample buffer supplemented with b-mercaptoethanol (control for plasma membrane remnants). Exosome preparations (70,000 x g pellets of cell culture media from 2 x 10^8 cells) were biotinylated as described above and solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. MHC class II was immunoprecipitated from a sample of biotinylated exosomes with the monoclonal

anti-class II antibody DA6.231 (19). The biotinylated cell membranes, biotinylated exosomes and immunoprecipitated MHC class II were analyzed by SDS-PAGE and Western blotting with ^{125}I-Streptavidin.--.

On page 4, lines 10 and 13, change "biotinilated" to --biotinylated--.

On page 4, line 12, replace "(18)" with --, as described above--.

On page 4, line 16, replace "(16)". with --, as described above--.

On page 4, line 23, before "Since", insert --The internal MIIC vesicles are formed by inward budding of the limiting membrane of MIICs (*see* Figures 16 and 17 in reference (6), similar to the process described for multivesicular bodies in other cell types (20))--.

On page 4, line 32, delete "(22)", and before "Both", insert the following text:

--The EBV-B cell lines RN (HLA-DR 15+) and JY (HLA DR15-) were incubated in the presence or absence of purified HSP 65 protein from *Mycobacterium Leprae* (50 μg/ml) (22) 4:71) for 4 hr in 10 ml serum free RPMI at 2 x 10^6 cells/ml, followed by the addition of 30 ml RPMI supplemened with 10% fetal calf serum (FCS) for 20 hr at 37°C. The cells were then washed to remove free antigen and incubated further for 24 hrs in RPMI/10% FCS medium at 37°C. Exosomes were prepared by differential centrifugation (Figure 2) and the efficiency of HSP 65 antigen presentation was measured by culturing 10,000 cells of the T cell clone 2F10 with irradiated (6000 rad) EBV cell. B cells or exosomes resuspended in 100 μl IMDM/10% pooled human serum were added to the T cell clone (50 μl IMDM/10% pooled human serum per well) in 96 well flatbottom microtiter plates (Costar, The Netherlands) for 4 days at 37°C, 5% CO_2 in humidified air. When indicated, 5 μg/ml of HLA-DR15 restricted epitope of HSP65 (peptide 418-427) was added to the exosomes. Sixteen hours before termination 0.5 μCi of [^{3}H]-thymidine was added to the wells. The cells were then harvested on glass fiber filters using an automatic cell harvester and the [^{3}H]-thymidine incorporation into cell DNA was determined by liquid scintillation counting. The results are expressed as the mean of triplicate measurements.--.

On page 4, line 32, start a new paragraph with the word "Both".

On page 4, line 36, delete "(23)" and before "A", insert the following text:

--As a control, exosomes were prepared from culture media of an equivalent amount of DR15-negative JY cells that have been incubated or not with antigen. JY cells secreted an equivalent amount of exosomes but these were ineffective in stimulating T cell proliferation.--.

On page 5, line 2, delete ", 24", and after ")." insert the following text:

--From these data exosomes appear to be 16 times less efficient in antigen presentation. However, in antigen presentation assays contact between B and T cells may be more efficient due to sedimentation of cells.--.

On page 5, line 8, delete "(25)", and after "themselves." insert the following text:

--Exosomes isolated from the culture medium of the murine B cell line TA3 (I-E^{k+}) incubated in the presence a RNase-derived peptide (aa 90-105) were also capable of stimulating IL2 secretion by WA.23 cells.--.

On page 5, lines 12 and 25, change "lysosomal" to --lysosomal--.

On page 5, line 24, delete "(28)" and before "However," insert the following text:

--A number of studies documented the presence of intact MHC class II molecules in 100,00 x g fractions from B cell culture media and their association with lipids (28). Our present observations shed new light on these data and suggest that the released MHC class II molecules were likely derived from secreted exosomes.--.

On page 5, line 26, change "lysosomes" to --lysosomes--.

On page 7, line 12, change "ecxipients" to --excipients--.

Delete the text on pages 8-15.

On a separate page following the claims, insert the following text for an abstract:

--The invention provides a novel vehicle for vaccination, in particular peptide vaccination. The new vehicle has been termed an exosome. Exosomes are vesicles derived from MHC class II enriched compartments in antigen presenting cells. The exosomes possess MHC II and/or MHC I molecules at their surface and possibly peptides derived from processed antigens in said MHC's. Thus the exosome is a perfect vaccination vehicle in that it presents the peptide in a natural setting. The peptides present in the exosome in the MHC molecule may be processed by the antigen presenting cell from which the exosome is derived.